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(54) Nicotianamine aminotrassferase and gene therefor

(57) A protein having an amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity, a gene encoding said protein as well as utilization thereof for enhancement of ability of absorbing insoluble iron in soil and for improvement of resistance to iron deficiency are provided.

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Description

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a nicotianamine aminotransferase, a gene therefor and utilization thereof.

Description of Related Art

Calcareous soil, a saline illuviation soil in dry ground, occupies about 30% of the soil in the world, including China, the Middle and Near East countries, the Central and North Africa, the Central and West America and so on. In this soil, iron in the soil is insolubilized due to a high pH. A plant can not grow in this soil, developing chlorosis by iron deficiency, unless it can absorb iron in soluble form from the root by any means. When agriculture and environmental afforestation are desired, measures against the deficiency of soluble iron in the soil will be an important problem.

As measures to solve the iron deficiency of plant by agricultural technique, it may be considered (1) to correct pH of the alkaline soil to neutral or slightly acidic one by addition of sulfur, (2) to apply a substance containing a chelated iron or (3) to increase soluble iron in the soil by enhancing coil microorganism activity, for example, by means of application of an organic substance, thereby increasing siderophore (an iron transporter) production by the microorganism.

These means for providing iron by soil treatment, however, are not always satisfactory because there are problems, for example, that a large amount of application material is required, that the effect is very unstable depending on the method of application including time of application, site of application, concentration, kind of spreader or the like and weather conditions. Therefore, development of novel techniques has been demanded.

Under these circumstances, the present inventors have conducted extensive studies and discovered a novel gene which is suitable for enhancing absorption ability on insoluble iron in soil and improving resistance to iron deficiency and thus have completed the present invention.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides:

(1) A protein comprising an amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity (hereinafter, referred to as the protein of the present invention),
(2) A gene encoding the protein as defined in the foregoing item 1 (hereinafter, referred to as the gene of the present invention),
(3) The gene in accordance with the foregoing item 2 having a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1 or 2,
(4) The gene in accordance with the foregoing item 3 having a nucleotide sequence represented by SEQ ID NO: 3 or 4,
(5) A plasmid comprising the gene in accordance with the foregoing item 2 (hereinafter, referred to as the plasmid of the present invention),
(6) An expression plasmid comprising (1) a promoter capable of functioning in a host cell, (2) the gene in accordance with the foregoing item 2 and (3) a terminator capable of functioning in a host cell, operably linked in the above described order (hereinafter, referred to as the expression plasmid of the present invention),
(7) A process for constructing an expression plasmid, which comprises combining (1) a promoter capable of functioning in a host cell, (2) the gene in accordance with the foregoing item 2 and (3) a terminator capable of functioning in a host cell, operably linked in the above described order (hereinafter, referred to as the process for construction of the present invention),,
(8) A transformant comprising a host cell harboring the plasmid as defined in foregoing item 5 or 6,
(9) The transformant in accordance with the foregoing item 8, wherein the host is a microorganism.
(10) The transformant in accordance with the foregoing item 8, wherein the host cell is a plant cell,
(11) A process for enhancing iron absorbing ability of a host cell, which comprises introducing into a host cell an expression plasmid formed by combining (1) a promoter capable of functioning in a host cell, (2) a nicotianamine aminotransferase gene and (3) a terminator capable of functioning in a host cell, operably linked in the above described order and transforming said host cell,
(12) The process in accordance with the foregoing item 11, wherein the host cell is a plant cell,
(13) The process in accordance with the foregoing item 12, wherein the gene of the nicotianamine aminotrans-

ferase is the gene as defined in the foregoing item 2,

(14) A gene fragment having a partial sequence of the gene in accordance with the foregoing item 2, 3 or 4 (hereinafter, referred to as the gene fragment of the present invention),

(15) The gene fragment in accordance with the foregoing item 14, wherein the number of the base is 15 or more and 50 or less,

(16) The gene fragment in accordance with the foregoing item 14 having the nucleotide sequence represented by SEQ ID NO: 5,

(17) A process for detecting a nicotianamine aminotransferase gene, which comprises detecting from plant gene fragments a nicotianamine aminotransferase gene having a nucleotide sequence encoding an amino acid sequence of an enzyme with the nicotianamine aminotransferase activity or a gene fragment thereof by applying the hybridization method using the gene fragment in accordance with the foregoing item 14, 15 or 16 (hereinafter, referred to as the process for detection of the present invention),

(18) A process for amplifying a nicotianamine aminotransferase gene, which comprises amplifying a nicotianamine aminotransferase gene having a nucleotide sequence encoding an amino acid sequence of an enzyme with the nicotianamine aminotransferase activity or a gene fragment thereof by applying PCR (polymerase chain reaction) on a plant gene fragment using the gene fragment as defined in the foregoing item 14, 15 or 16 as a primer (hereinafter, referred to as the process for amplification of the present invention),

(19) A process for obtaining a nicotianamine aminotransferase gene, which comprises identifying a nicotianamine aminotransferase gene or a gene fragment thereof by the process as defined in the foregoing item 17 or 18, and isolating and purifying the identified gene or the gene fragment thereof, and

(20) A nicotianamine aminotransferase gene obtained by the process as defined in the foregoing item 19.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be described below in more detail.

The protein of the present invention comprises the amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity.

Such protein can be prepared from Gramineae plants, for example, barley (*Hordeum vulgare*) or the like by a process, for example, a process described below.

Examples of the protein of the present invention include an amino acid sequence of SEQ ID NO: 1 or 2 or an amino acid sequence having a molecular weight of 47 kDa comprising 429 amino acids beginning from the amino acid of NO: 33 in SEQ ID NO: 1.

The nicotianamine aminotransferase activity hereinafter refers to an ability of transferring an amino group from nicotianamine to 2-oxoglutarate.

The nicotianamine aminotransferase activity can be measured by, for example, a method described in Kanazawa, K et al., Journal of Experimental Botany, 45, 1903 - 1906 (1994) and others. Specifically, substrates nicotianamine, 2-oxoglutaric acid, and pyridoxal phosphate as a coenzyme are added to an enzyme solution and the mixture is reacted at 25°C for 30 minutes. After the reaction, the reaction product is reduced by adding $NaBH_3$ and deoxymugineic acid is determined by HPLC.

In order to prepare the protein or the present invention from a Gramineae plant such as barley (*Hordeum vulgare*) or the like, for example, whole root of a Gramineae plant such as barley or the like treated for iron deficiency is triturated and the protein of the present invention is partly purified by subjecting the obtained extract to hydrophobic interaction chromatography, adsorption chromatography, anion exchange chromatography, gel filtration, and second adsorption chromatography in this order using the activity as an indicator. The individual protein fraction obtained from the second adsorption chromatography is subjected to two-dimensional electrophoresis and protein spots are detected which rises and tails in proportion to the intensity of nicotianamine aminotransferase activity of each fraction. The detected spots indicate the protein of the present invention. The protein of the present invention can be purified by isolating from the two-dimensional electrophoresis gel.

Mugineic acid analogues such as deoxymugineic acid produced by a reaction catalyzed by the protein of the present invention and a subsequent reduction reaction, mugineic acid and 3'-hydroxymugineic acid produced by a still subsequent hydroxylation reaction, or the like, solubilizes iron by forming a chelate complex with insoluble iron in the soil. Some kind of plants can biosynthesize said mugineic acid analogues, which are secreted from their root to the soil in the rooting zone, thereby solubilizing insoluble iron in the form of a mugineic acid complex and absorbing the iron complex directly through the root. Therefore, it is possible to enhance production of mugineic acid analogues and increase ability of absorbing insoluble iron by appropriately expressing a large amount of the protein of the present invention in said plants.

The gene of the present invention encodes a protein comprising the amino acid sequence represented by SEQ ID

NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity.

Such gene can be prepared from Gramineae plants, for example, barley (*Hordeum vulgare*) or the like by a process, for example, a process described below.

Further, the gene of the present invention includes a gene encoding a protein comprising the amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity and encompasses a gene, for example, that hybridizes with the said gene sequence under stringent conditions. The stringent conditions herein refer to conditions used, for example, in the screening of cDNA library described in Example 4.

Specific examples of the nucleotide sequence of the gene include the nucleotide sequence represented by SEQ ID NO: 3 (the loci of CDS being 62 - 1444) or SEQ ID NO: 4 (the loci of CDS being 76 - 1731).

It is possible to increase ability of absorbing insoluble iron in the soil in the rooting zone and improve resistance to iron deficiency by introducing the gene of the present invention into a plant which absorbs iron making use of mugineic acid compounds thereby enhancing biosynthesizing ability of mugineic acid compounds in the obtained transformant plant.

In order to prepare the gene of the present invention, for example, the amino acid sequence of peptide fragments obtained by partially hydrolyzing the protein of the present invention and the N-terminal amino acid sequence of the protein of the present invention are determined by a protein sequencer. Two or more primers comprising DNA sequences expected from these amino acid sequences are synthesized. By conducting PCR using as a template a cDNA synthesized from mRNA prepared from the root of a Gramineae plant such as barley treated for iron deficiency by means of a reverse transcriptase, cDNA fragment of the gene of the present invention is amplified. Using the amplified cDNA fragment as a probe, screening of cDNA library described below is performed. A cDNA is synthesized from mRNA Prepared from the root of a Gramineae plant such as barley treated for iron deficiency by means of a reverse transcriptase and this is integrated into a phage vector such as lambda ZAPII or the like or a plasmid vector such as pUC or the like to prepare a cDNA library. This library is screened using the above-mentioned probe and a cDNA of the nicotianamine aminotransferase gene is selected. The selected cDNA can be confirmed to be that of the nicotianamine aminotransferase gene (cDNA of the gene of the present invention) by determining the sequence of the selected cDNA.

In order to obtain genome DNA using the cDNA selected in this manner and determine its sequence, for example, plant tissue such as leaf, stem, root or the like is instantly frozen and sufficiently triturated with a mortar and pestle or a Waring blender. The genome DNA is extracted from the obtained triturated product according to the ordinary method as described in Itaru Watanabe (supervisor), Masahiro Sugiura (editor), "Cloning and Sequencing (a manual for experiment of plant biotechnology)", Nosonbunka-sha, Tokyo (1989) or the like. The obtained genome DNA is digested with an appropriate restriction enzyme and the obtained genome DNA fragments are fractionated by a known method such as sucrose density gradient centrifugation or cesium chloride equilibrium centrifugation or the like. Each of the genome DNA fragment fractions is subjected to normal Southern hybridization using the selected cDNA (cDNA of the gene of the present invention) as a probe to decide a genome DNA fragment fraction containing the desired gene.

A genome DNA library is prepared by ligating the genome DNA fragment fraction to a commercially available vector such as plasmid, phage, cosmid or the like. The library is subjected to normal screening by hybridization using the cDNA of the gene of the present invention as a probe to obtain a genome DNA clone containing a nucleotide sequence encoding the amino acid sequence of the protein of the present invention. The obtained DNA clone can be subcloned to a vector, for example, plasmid or the like suitable for analysis of gene sequence and the sequence is analyzed according to a routine method to determine the sequence of the genome DNA containing a sequence encoding the amino acid sequence of the protein of the present invention.

The transcription initiation site of genome DNA of the gene of the present invention can be determined by the primer extension method described in Bina-Stem, Met et al., Proc. Natl. Acad. Sci. USA, 76, 731 (1979), Sollner-Webb and Reeder, R. H., Cell, 18, 485 (1979) or the like or the S1 mapping method described in Berk, A. J. and Sharp, P. A., Proc. Natl. Acad. Sci. USA, 75, 1274 (1978). A TATA sequence necessary for the transcription initiation is present in the upstream of the transcription initiation site decided in this manner. A promoter sequence bearing control of gene expression is present usually at 1 kb to about 10 kb upstream of this transcription initiation site. The promoter region of the gene of the present invention can be finally determined, for example, by connecting gene fragments having promoter regions of various length with a reporter gene such as GUS or the like, preparing transgenic plants into which the connected product are introduced, and studying presence or absence of expression of the reporter gene in various tissues of the prepared plants.

On the other hand, a terminator sequence is present in the genome DNA region corresponding to a poly-A sequence usually present in the downstream of a poly(A) addition signal (consensus sequence being AATAAA) which exists in a terminal 3'-nontranslation region at the downstream of termination codon, and has an effective translation terminating function.

The plasmid of the present invention contains a gene encoding a protein comprising the amino acid sequence rep-

resented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity.

Preferred specific examples of the plasmid include a plasmid prepared by cloning a nicotianamine aminotransferase gene having a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1 into pSK- (Strategene). This has a characteristic that its vector portion is small, it has a great number of copies in *Escherichia coli*, and thus it is suitable for preparation of DNA or analysis of DNA structure.

The expression plasmid of the present invention can be constructed by combining (1) a promoter capable of functioning in a host cell, (2) the gene encoding a protein comprising the amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity and (3) a terminator capable of functioning in a host cell, operably linked in the above described order.

The expression "operably linked" used hereinafter means that, when the constructed plasmid is introduced into a host cell to transform it, the gene of the present invention is integrated under the control of a promoter such that the gene has a function of expressing the protein of the present invention in said host cell.

The promoter capable of functioning in a host cell includes, for example, *Escherichia coli* lactose operon promoter, yeast alcohol dehydrogenase (ADH) promoter, adenovirus major late (Ad. ML) promoter, SV40 early promoter, baculovirus promoter and the like. When the host cell is a plant cell, the promoter includes, for example, T-DNA derived constitutive promoters such as nopaline synthase gene (NOS) promoter, octopine synthase gene (OCS) promoter and the like, plant virus derived promoters such as cauliflower mosaic virus (CaMV) derived 19S and 35S promoters and the like, and inducible promoters such as phenylalanine ammonialyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter, pathogen-related (PR) gene promoter and the like. Further, it includes known plant promoters not limited to them.

The terminator capable of functioning in a host cell includes, for example, yeast HIS terminator sequence, ADH1 terminator, SV40 early splicing region and the like. When the host cell is a plant cell, the terminator includes, for example, T-DNA derived constitutive terminators such as nopaline synthase gene (NOS) terminator and the like, plant virus derived terminators such as garlic virus GV1, GV2 terminators and the like. Further, it includes known plant terminators not limited to them.

A host cell is transformed by introducing such plasmid ((expression) plasmid of the present invention) into said host cell. When the hoot cell is a plant cell, the (expression) plasmid of the present invention is introduced into a plant cell by any of conventional means such as Agrobacterium infection method (JP-B-2-58917 and JP-A-60-70080), electroporation method into protoplast (JP-A-60-251887 and JP-A-5-68575), particle gun method (JP-A-508316 and JP-A-63-258525) and the like, and a transformed plant cell can be obtained by selecting a plant cell into which the gene of the present invention is introduced. The transformed plant body is obtained by regenerating a plant body according to a conventional plant cell culturing process, for example, described in Hirohumi Utimiya, Manual for Plant Gene Manipulation (Method for Producing Transgenic Plants), Published by Kodansha Scientific (ISBN 4-06-153515-7 C3045), 1990, pages 27 - 55.

By introducing the plasmid of the present invention into host cells which are any kind of microorganism such as *Escherichia coli* or the like and allowing high expression in said host cells, a large amount of the protein of the present invention can easily be isolated from the host cells. A screening system for inhibitors to nicotianamine aminotransferase activity constructed by utilizing the mass produced protein of the present invention. For example, according to the process for measuring nicotianamine aminotransferase activity described above, substrates nicotianamine, 2-oxoglutaric acid and pyridoxal phosphate as the coenzyme as well as a candidate inhibitor compound are added to the prepared enzyme solution, and the mixture is reacted at 25°C for 30 minutes. After the reaction, compounds shoving no nicotianamine aminotransferase activity are selected by reducing the reaction product with addition of $NaBH_3$ and deoxymugineic acid by HPLC.

In plants absorbing iron utilizing mugineic acid compounds, expression of the nicotianamine aminotransferase gene is strongly induced in iron deficiency conditions. Since the common soil (upland soil) is under the oxidative conditions and the ferric iron concentration in soil solution is only a level extremely lower than 10^{-4} - 10^{-8} M that is required by plants, nicotianamine aminotransferase gene and mugineic acid biosynthesis gene are always strongly induced. In other words, plants positively absorb insoluble iron by routinely biosynthesizing mugineic acid compounds and secreting them from the root to the soil in the rooting zone.

The inhibitors to nicotianamine aminotransferase activity selected by the screening system may be compounds useful as selective herbicides against plants that absorb iron by utilizing compounds analogous to mugineic acid.

Further, the present invention provides a process for enhancing iron absorbing ability, which comprises introducing in a host cell an expression plasmid formed by combining (1) a promoter capable of functioning in a host cell, (2) a nicotianamine aminotransferase gene and (3) a terminator capable of functioning in a host cell, operably in the above described order and transforming said host cell. The promoter capable of functioning in a host cell includes the promoters as described above.

The nicotianamine aminotransferase gene includes, for example, a plant derived nicotianamine aminotransferase gene and preferably the gene of the present invention.

The terminator capable of functioning in a host cell includes the terminators as described above.

The gene fragment of the present invention refers to a gene fragment having a partial sequence of the gene of the present invention represented by SEQ ID NO'3 or 4 and includes a gene fragment having a partial sequence of the gene encoding a protein comprising the amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity, specifically, for example, a gene fragment represented by SEQ ID NO: 5.

These gene fragments are useful as probes in hybridization or primers in PCR. Particularly, as primers used in PCR, a gene fragment having 15 or more and 50 or less nucleotides are preferred.

The process for detection of the present invention is a process in which a nicotianamine aminotransferase gene having a nucleotide sequence encoding an amino acid sequence of an enzyme with the nicotianamine aminotransferase activity or a gene fragment thereof is detected from plant gene fragments by applying the hybridization method using the gene fragment of the present invention as a probe.

Specifically, for example, the process can be performed according to the method described in "Molecular Cloning: A Laboratory Manual, 2nd edition" (1989), Cold spring Harbor Laboratory Press or in "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN0-471-50338-X. The gene fragments used here may include, for example, cDNA library, genome DNA library or the like of the targeted plant. Said plant gone fragments may be a commercially available library as such derived from a plant, or may also be a library prepared according to the conventional method for preparing a library described in "Molecular Cloning: A Laboratory Manual, 2nd edition" (1989), Cold Spring Harbor Laboratory Press or in "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN0-471-50338-X.

It can also be possible to obtain nicotianamine aminotransferase gene by identifying the nicotianamine aminotransferase gene or a fragment thereof according to the process for detection of the present invention and isolating/purifying the identified gene or gene fragment.

The process for detection of the present invention may be utilized in analysis of plants. Specifically, a plant genome DNA is prepared from different cultivars of a specific plant species according to the process for detection of the present invention the ordinary method described in Itaru Watanabe (supervisor), Masahiro Sugiura (editor), "Cloning and Sequencing (a manual for experiment of plant biotechnology)", Nosonbunka-sha, Tokyo (1989) or the like. It is then incised with at least several kinds of suitable restriction enzymes, electrophoresed, and used for preparing a filter by brotting according to the ordinary method.

Hybridization is conducted on the filter using a probe prepared by the ordinary method and differences in phenotype character accompanied by mugineic acid biosynthesis between cultivars based on the difference in length of DNA fragments. Further, a plant is decided to be a recombinant gene plant if the plant has a greater number of detected hybridization bands than a non-recombinant gene plant when the specific plant is compared with the non-recombinant plant. This method is preferably carried out according to the RFLP (Restriction Fragment Length Polymorphism) method described in Ko Shimamoto and Takuji Sasaki (supervisors), "Protocols for PCR Experiment on Plants", Shujunsha, Tokyo (1995), ISBN4-87962-144-7, pp 90 - 94.

The process for amplification of the present invention is a process in which a nicotianamine aminotransferase gene having a nucleotide sequence encoding an amino acid sequence of an enzyme with the nicotianamine aminotransferase activity or a gene fragment thereof is amplified by applying PCR (polymerase chain reaction) on a plant gene fragments using the gene fragment of the present invention as a primer. Specifically, for example, the process can be performed according to the method described in Ko Shimamoto and Takuji Sasaki (supervisors), "Protocols for PCR Experiment on Plants", Shujunsha, Tokyo (1995), ISBN4-87962-144-7 or the like.

It can also be possible to obtain nicotianamine aminotransferase gene by identifying the nicotianamine aminotransferase gene or a fragment thereof according to the process for amplification of the present invention and isolating/purifying the identified gene or gene fragment.

Further, the process for amplification of the present invention may be utilized in analysis of plants. Specifically, for example, a part or the whole of the gene of the present invention is amplified by conducting PCR using a plant genome DNA prepared from a specific plant species as a template and the gene fragment of the present invention as a primer. The obtained PCR product is mixed with a formaldehyde solution and the mixture is denatured by heating at 85°C for 5 minutes, followed by rapid cooling on ice. This sample is electrophoresed on, for example, 6% acrylamide gel containing glycerol at a concentration of 0% or 10%. The electrophoresis is carried out with a commercially available electrophoresis apparatus for SSCP (Single Strand Conformation Polymorphism) keeping the gel temperature at, for example, 5°C, 25°C, 37°C and so on. The migrated gel in subjected to ethidium bromide staining or the like using a commercially available reagent to detect DNA.

Differences in phenotype character accompanied by mugineic acid biosynthesis between cultivars based on mutation in the gene of the present invention is analyzed from the differences in migration of the DNA fragments detected.

This method is preferably carried out according to the method described in Ko Shimamoto and Takuji Sasaki (supervisors), "Protocols for PCR Experiment on Plants", Shujunsha, Tokyo (1995), ISBN4-87962-144-7, pp 141 - 146. EXAMPLES

The present invention will now be described in more detail on the bases of Examples, which should not be construed as a limitation upon the scope of the present invention.

Example 1 (Method of Isolating the protein of the present invention)

In an extraction buffer solution (0.2 M Tris-HCl, 10 mM EDTA, 0.1 mM p~APMSF, 10 mM DTT, 5% glycerol, 5% polyvinyl pyrrolidone, pH 8.) was triturated 150 g of root of barley treated for iron deficiency. The trituration product was centrifuged at 8,000 x g for 30 minutes and the supernatant was separated. Ammonium sulfate was added to the obtained supernatant until 30% saturation was attained. The produced sample was applied over Butyl Toyopearl (manufactured by Toso) equilibrated with 30% saturated ammonium sulfate buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM DTT), and eluted with 15% saturated ammonium sulfate buffer after washing with the former buffer. To eluted fractions was added p-APMSF at a final concentration of 0.1 mM and the mixture was dialyzed overnight against 0.1 mM KCl, 50 mM KH_2PO_4/K_2HPO_4 (pH 6.8), 10 mM DTT, followed by application over Hydroxylapatite (100 - 350 mesh, manufactured by Nakarai) equilibrated with said buffer. Then it was washed with the same buffer and eluted with 0.5 M KH_2PO_4/K_2HPO_4 (pH 6.8), 10 mM DTT. The eluted fractions were treated with Molecut (Millipore, differential molecular weight 10,000) in order to exchange buffer with 20 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM DTT and applied over DEAE Sephasel (manufactured by Pharmacia) equilibrated with the same buffer. After washing with the same buffer, it was eluted with 10 mM - 500 mM KCl concentration gradient. Non-adsorbed fractions from DEAE Sephasel were treated with Molcut in order to exchange buffer with 20 mM Tris-HCl (pH 8.0), 10 mM KCl, 5 mM EDTA, 1 mM DTT and applied over NA-Sepharose 4B which was EAH-Sepharose 4B (manufactured by Pharmacia) having bound nicotianamine (NA). After washing with the same buffer, it was eluted with 1 mM NA, 10 mM KCl, 20 mM Tris-HCl (pH 6.0). The eluted fractions were subjected to two-dimensional electrophoresis, which allowed very concentrated spot as compared with the sample before applying on NA-Sepharose 4B column. The spot indicated the protein of the present invention, which was isolated by separating said spot.

The N-terminal amino acid sequence of the protein of the present invention as separated was analyzed by a protein sequencer (manufactured by Applied Biosystems). The result showed revealed an amino acid sequence shown by the amino acids of Nos 33 to 47 in the seq. ID NO.1. Further, N-terminal amino acid sequences for 3 peptide fragments formed by treating it with 70% formic acid solution containing 1% bromocyan were analyzed in the same manner.

Example 2 (Preparation of a probe for cloning of cDNA of the protein of the present invention)

From 6g of root of barley treated for iron deficiency 255 μg of whole RNA was recovered according to the SDS-phenol method described in Itaru Watanabe (supervisor), Masahiro Sugiura (editor), "Cloning and Sequencing (a manual for experiment of plant biotechnology)", Nosonbunka-sha, Tokyo (1989), pp 34 - 40. From the recovered whole RNA, 75 μg portion was taken and used to prepare poly(A)+RNA using Dynabeads mRNA Purification Kit (manufactured by Dynal). The prepared poly(A)+RNA was reverse transcribed with dT17 adapter primer (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3') to prepare cDNA. A part of the prepared cDNA was used for amplification of cDNA fragment of the gene of the present invention by two steps PCR. In the first reaction, PCR was conducted with a primer 1 (5'-GCIGTIGARTGGAAYTTYGCIMG-3') synthesized on the basis of N-terminal amino acid sequence of the protein of the present invention and the above described dT17 adapter primer and using the obtained cDNA as a template at 94°C (40 seconds), 40°C (1 minute), and 72°C (2 minutes), repeated by 25 cycles, and at 94°C (40 seconds), 45°C (1 minute), and 72°C (2 minutes), repeated by 25 cycles. Using this PCR reaction solution as a template, the second PCR was conducted with a primer 2 (5'-GCDATRTGICCRAAIACICC-3') synthesized on the basis of N-terminal amino acid sequence of the peptide fragment formed by treating with 70% formic acid solution containing 1% bromocyan as described above and the primer 1 at 94°C (40 seconds), 45°C (1 minute), and 72°C (2 minutes), repeated by 40 cycles. The DNA fragment of about 600 bp amplified by the second PCR was purified by excising from 0.8% agarose electrophoresis gel and used as a probe for screening cDNA library.

Example 3 (Preparation of cDNA library from root of barley treated for iron deficiency)

Using a commercially available cDNA synthesis kit (Super Script (trademark) Plasmid System for cDNA Synthesis and Plasmid Cloning, manufactured by Gibco BRL), cDNA was synthesized from 5 μg of poly(A)+RNA prepared from root of barley treated for iron deficiency described in Example 2. The product was ligated with SalI adapter and incised with NotI to recover cDNA.

A vector for cDNA library (hereinafter, referred to as pYH23) was prepared by adding some modification to yeast

multi-copy plasmid YEplac181 described in R. Daniel Gietz and Akio Sugino, Gene, 74 (1988), pp 527-534. Specifically, HindIII and BamHI to EcoRI site in the multi-cloning site of YFplac181 was eliminated. Further, promoter and terminator sequences of alcohol dehydrogenase derived from pTV-100 were subcloned at SphI site, and NotI linker was inserted at BamHI site of this fragment.

The pYH23 prepared in this manner was digested with NotI and XhoI, after inserting cDNA prepared as above, *Escherichia coli* XL1-Blue strain was transformed to provide cDNA library derived from 300,000 independent colonies.

Example 4 (Screening of cDNA clones of the present invention)

A probe DNA for cDNA cloning of the protein of the present invention was prepared by radioactively labeling the probe prepared in Example 3 with a commercially obtainable radioactivity label kit (Random Primer DNA Labeling Kit Ver. 2, TaKaRa). *Escherichia coli* having a plasmid DNA of cDNA library derived from root of barley treated for iron deficiency as prepared in Example 3 was inoculated in LB medium, incubated at 37°C for 10 hours, and then transferred to a commercially available Nylon membrane (Hybond (trademark)-N+, Amersham Life Science). The membrane was treated with 10% SDS for 3 minutes, an alkaline denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, a neutralizing solution (0.5 M Tris-HCl (pH 7.0), 1.5 M NaCl) for 3 minutes, 2 x SSPE (20 mM phosphate buffer (pH 7.4), 0.3 M NaCl, 5 mM EDTA) twice for 3 minutes, dried, and irradiated with ultraviolet rays for 3 minutes to irreversibly fix DNA on the membrane. Prehybridization was carried out at 65°C for 1 hour using a prehybridization solution (5 x Denhart's solution, 5 x SSPE, 0.1% SDS, 100 μg/ml denatured salmon testis DNA). Then, hybridization as carried out in a solution having the radioactively labeled probe added to a hybridization solution (5 x Denhart's solution, 5 x SSPE, 0.1% SDS) at 65°C for 12 hours. Thereafter, the membrane was washed once with 6 x SSP at 65°C for 10 minutes, twice with 2 x SSP, 0.1% SDS at 42°C for 10 minutes, and exposed to Fuji Medical X-ray Film to detect positive colonies. Second and third screenings were performed in the same manner and cDNA clone of the protein of the present invention was isolated.

Example 5 (Determination of nuceotide sequence of cDNA encoding the protein of the present invention)

The cDNA clone of the protein of the present invention isolated in Example 4 was subcloned in a plasmid vector pBluescript SK(-) according to the conventional method described in J. Sambrook, E. F. Fritsh, T. Maniatis, "Molecular Cloning, Second Edition" Cold Spring Harbor Press (1989) to give a plasmid cDNA clone. Nucleotide sequence (SEQ. ID NO. 3 and 4) of the insert in said cDNA clone was determined (1) by 373A DNA Sequencer manufactured by Applied Biosystems using Tag Dye Primer Cycle Sequencing Kit (manufactured by Applied Biosystems), (2) by DSQ-1000L DNA Sequencer (manufactured by Shimadzu) using Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit (manufactured by Amersham Life Science), or (3) by BAS-2000 (manufactured by Fuji Film) using BcaBEST (trademark) Dideoxy Sequencing Kit (manufactured by TaKaRa). The total amino acid sequences of the protein (see SEQ ID NO: 1 and 2) were determined from the sequence (see SEQ ID NO: 3 and 4). The protein of the SEQ ID NO: 1 had 461 amino acids and its molecular weight was calculated to be 49564.15, and the protein of the SEQ ID NO: 1 had 551 amino acids and its molecular weight was calculated to be 58148.62, According to the present invention, it could be possible to provide a novel nicotianamine aminotransferase, a gene therefor and so on.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Sumitomo Chemical Company, Limited

(ii) TITLE OF INVENTION: Nicotianamine Aminotransferase and Gene therefor

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:

(A) ADRESSEE: Sumitomo Chemical Company, Limited

(B) STREEET: 5-33, Kitahama 4-Chome, Chuo-ku

(C) CITY: Osaka

(D) STATE:Osaka-fu

(E) COUNTRY: Japan

(F) ZIP: 541-0858

(G) TELEPHONE: 81-6-220-3405

(H) TELEFAX: 81-6-220-3390

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 1.4MB

(B) COMPUTER: IBM

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: Word 6.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:19.2.1998

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:JP 09-037499

(B) FILING DATE:21.02.1997

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME:KWP

(B) REGISTRATION NUMBER: Association Nr. 5

(C) REFERENCE/DOCKET NUMBER: SM1009

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE:08161/930-211

(B) TELEFAX:08161/930-100

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISICS:

(A) LENGTH: 461 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Val His Gln Ser Asn Gly His Gly Glu Ala Ala Ala Ala Ala Ala
 1               5                  10                  15
Asn Gly Lys Ser Asn Gly His Ala Ala Ala Asn Gly Lys Ser Asn
            20                  25                  30
Gly His Ala Ala Ala Ala Val Glu Trp Asn Phe Ala Arg Gly Lys
        35                  40                  45
Asp Gly Ile Leu Ala Thr Thr Gly Ala Lys Asn Ser Ile Arg Ala Ile
    50                  55                  60
Arg Tyr Lys Ile Ser Ala Ser Val Glu Glu Ser Gly Pro Arg Pro Val
65                  70                  75                  80
Leu Pro Leu Ala His Gly Asp Pro Ser Val Phe Pro Ala Phe Arg Thr
                85                  90                  95
Ala Val Glu Ala Glu Asp Ala Val Ala Ala Ala Leu Arg Thr Gly Gln
            100                 105                 110
Phe Asn Cys Tyr Ala Ala Gly Val Gly Leu Pro Ala Ala Arg Ser Ala
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        115                 120                 125
Val Ala Glu His Leu Ser Gln Gly Val Pro Tyr Lys Leu Ser Ala Asp
    130                 135                 140
Asp Val Phe Leu Thr Ala Gly Gly Thr Gln Ala Ile Glu Val Ile Ile
145                 150                 155                 160
Pro Val Leu Ala Gln Thr Ala Gly Ala Asn Ile Leu Leu Pro Arg Pro
                165                 170                 175
Gly Tyr Pro Asn Tyr Glu Ala Arg Ala Ala Phe Asn Lys Leu Glu Val
            180                 185                 190
Arg His Phe Asp Leu Ile Pro Asp Lys Gly Trp Glu Ile Asp Ile Asp
        195                 200                 205
Ser Leu Glu Ser Ile Ala Asp Lys Asn Thr Thr Ala Met Val Ile Ile
    210                 215                 220
Asn Pro Asn Asn Pro Cys Gly Ser Val Tyr Ser Tyr Asp His Leu Ala
225                 230                 235                 240
Lys Val Ala Glu Val Ala Arg Lys Leu Gly Ile Leu Val Ile Ala Asp
                245                 250                 255
Glu Val Tyr Gly Lys Leu Val Leu Gly Ser Ala Pro Phe Ile Pro Met
            260                 265                 270
Gly Val Phe Gly His Ile Ala Pro Val Leu Ser Ile Gly Ser Leu Ser
        275                 280                 285
Lys Ser Trp Ile Val Pro Gly Trp Arg Leu Gly Trp Val Ala Val Tyr
    290                 295                 300
Asp Pro Thr Lys Ile Leu Glu Lys Thr Lys Ile Ser Thr Ser Ile Thr
305                 310                 315                 320
Asn Tyr Leu Asn Val Ser Thr Asp Pro Ala Thr Phe Val Gln Glu Ala
                325                 330                 335
Leu Pro Lys Ile Leu Glu Asn Thr Lys Ala Asp Phe Phe Lys Arg Ile
            340                 345                 350
Ile Gly Leu Leu Lys Glu Ser Ser Glu Ile Cys Tyr Arg Glu Ile Lys
        355                 360                 365
Glu Asn Lys Tyr Ile Thr Cys Pro His Lys Pro Glu Gly Ser Met Phe
    370                 375                 380
Val Met Val Lys Leu Asn Leu His Leu Leu Glu Glu Ile His Asp Asp
385                 390                 395                 400
Ile Asp Phe Cys Cys Lys Leu Ala Lys Glu Glu Ser Val Ile Leu Cys
                405                 410                 415
Pro Gly Ser Val Leu Gly Met Glu Asn Trp Val Arg Ile Thr Phe Ala
            420                 425                 430
Cys Val Pro Ser Ser Leu Gln Asp Gly Leu Glu Arg Val Lys Ser Phe
        435                 440                 445
Cys Gln Arg Asn Lys Lys Lys Asn Ser Ile Asn Gly Cys
    450                 455                 460 461
```

(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISICS:

(A) LENGTH: 551 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ala Thr Val Arg Gln Ser Asp Gly Val Ala Ala Asn Gly Leu Ala
  1               5                  10                  15
Val Ala Ala Ala Ala Ala Asn Gly Lys Ser Asn Gly His Gly Val Ala Ala
            20                  25                  30
Ala Val Asn Gly Lys Ser Asn Gly His Gly Val Asp Ala Asp Ala Asn
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                35                  40                  45
Gly Lys Ser Asn Gly His Gly Val Ala Ala Asp Ala Asn Gly Lys Ser
            50                  55                  60
Asn Gly His Ala Glu Ala Thr Ala Asn Gly His Gly Glu Ala Thr Ala
65                  70                  75                  80
Asn Gly Lys Thr Asn Gly His Arg Glu Ser Asn Gly His Ala Glu Ala
                85                  90                  95
Ala Asp Ala Asn Gly Glu Ser Asn Glu His Ala Glu Asp Ser Ala Ala
            100                 105                 110
Asn Gly Glu Ser Asn Gly His Ala Ala Ala Ala Ala Glu Glu Glu Glu
        115                 120                 125
Ala Val Glu Trp Asn Phe Ala Gly Ala Lys Asp Gly Val Leu Ala Ala
    130                 135                 140
Thr Gly Ala Asn Met Ser Ile Arg Ala Ile Arg Tyr Lys Ile Ser Ala
145                 150                 155                 160
Ser Val Gln Glu Lys Gly Pro Arg Pro Val Leu Pro Leu Ala His Gly
                165                 170                 175
Asp Pro Ser Val Phe Pro Ala Phe Arg Thr Ala Val Glu Ala Glu Asp
            180                 185                 190
Ala Val Ala Ala Ala Val Arg Thr Gly Gln Phe Asn Cys Tyr Pro Ala
        195                 200                 205
Gly Val Gly Leu Pro Ala Ala Arg Ser Ala Val Ala Glu His Leu Ser
    210                 215                 220
Gln Gly Val Pro Tyr Met Leu Ser Ala Asp Asp Val Phe Leu Thr Ala
225                 230                 235                 240
Gly Gly Thr Gln Ala Ile Glu Val Ile Ile Pro Val Leu Ala Gln Thr
                245                 250                 255
Ala Gly Ala Asn Ile Leu Leu Pro Arg Pro Gly Tyr Pro Asn Tyr Glu
            260                 265                 270
Ala Arg Ala Ala Phe Asn Arg Leu Glu Val Arg His Phe Asp Leu Ile
        275                 280                 285
Pro Asp Lys Gly Trp Glu Ile Asp Ile Asp Ser Leu Glu Ser Ile Ala
    290                 295                 300
Asp Lys Asn Thr Thr Ala Met Val Ile Ile Asn Pro Asn Asn Pro Cys
305                 310                 315                 320
Gly Ser Val Tyr Ser Tyr Asp His Leu Ser Lys Val Ala Glu Val Ala
                325                 330                 335
Lys Arg Leu Gly Ile Leu Val Ile Ala Asp Glu Val Tyr Gly Lys Leu
            340                 345                 350
Val Leu Gly Ser Ala Pro Phe Ile Pro Met Gly Val Phe Gly His Ile
        355                 360                 365
Thr Pro Val Leu Ser Ile Gly Ser Leu Ser Lys Ser Trp Ile Val Pro
    370                 375                 380
Gly Trp Arg Leu Gly Trp Val Ala Val Tyr Asp Pro Arg Lys Ile Leu
385                 390                 395                 400
Gln Glu Thr Lys Ile Ser Thr Ser Ile Thr Asn Tyr Leu Asn Val Ser
                405                 410                 415
Thr Asp Pro Ala Thr Phe Ile Gln Ala Ala Leu Pro Gln Ile Leu Glu
            420                 425                 430
Asn Thr Lys Glu Asp Phe Phe Lys Ala Ile Ile Gly Leu Leu Lys Glu
        435                 440                 445
Ser Ser Glu Ile Cys Tyr Lys Gln Ile Lys Glu Asn Lys Tyr Ile Thr
    450                 455                 460
Cys Pro His Lys Pro Glu Gly Ser Met Phe Val Met Val Lys Leu Asn
465                 470                 475                 480
Leu His Leu Leu Glu Glu Ile Asp Asp Asp Ile Asp Phe Cys Cys Lys
                485                 490                 495
Leu Ala Lys Glu Glu Ser Val Ile Leu Cys Pro Gly Ser Val Leu Gly
```

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            500                 505                 510
Met Ala Asn Trp Val Arg Ile Thr Phe Ala Cys Val Pro Ser Ser Leu
        515                 520                 525
Gln Asp Gly Leu Gly Arg Ile Lys Ser Phe Cys Gln Arg Asn Lys Lys
    530                 535                 540
Arg Asn Ser Ser Asp Asp Cys
545                 550 551
```

(4) INFORMATION FOR SEQ ID NO: 3:
  (i) SEQUENCE CHARACTERISICS:
    (A) LENGTH: 1660 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY:
  (ii) MOLECULAR TYPE: cDNA to mRNA
  (iii) HYPOTHETICAL:
(iv) ANTI-SENSE:
(v) FEATURE: CDS
(vi) LOCATION: 62 .. 1447
(vii) IDENTIFICATION METHOD: P
  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
ATTGACTAGC TAGTTCATTC CCTGCCACAC TGCTAGTACT CCTCCTCGTT TCCTCGTGGC     60
A ATG GTA CAC CAG AGC AAC GGC CAC GGC GAG GCC GCC GCC GCC GCC         106
  Met Val His Gln Ser Asn Gly His Gly Glu Ala Ala Ala Ala Ala
    1               5                  10                  15
GCC AAC GGC AAG AGC AAC GGG CAC GCC GCC GCC GCG AAC GGC AAG AGC      154
Ala Asn Gly Lys Ser Asn Gly His Ala Ala Ala Ala Asn Gly Lys Ser
                20                  25                  30
AAC GGG CAC GCG GCG GCG GCG GTG GAG TGG AAT TTC GCC CGG GGC      202
Asn Gly His Ala Ala Ala Ala Ala Val Glu Trp Asn Phe Ala Arg Gly
            35                  40                  45
AAG GAC GGC ATC CTG GCG ACG ACG GGG GCG AAG AAC AGC ATC CGG GCG      250
Lys Asp Gly Ile Leu Ala Thr Thr Gly Ala Lys Asn Ser Ile Arg Ala
        50                  55                  60
ATA CGG TAC AAG ATC AGC GCG AGC GTG GAG GAG AGC GGG CCG CGG CCC      298
Ile Arg Tyr Lys Ile Ser Ala Ser Val Glu Glu Ser Gly Pro Arg Pro
    65                  70                  75
GTG CTG CCG CTG GCC CAC GGT GAC CCG TCC GTG TTC CCG GCC TTC CGC      346
Val Leu Pro Leu Ala His Gly Asp Pro Ser Val Phe Pro Ala Phe Arg
 80                  85                  90                  95
ACG GCC GTC GAG GCC GAA GAC GCC GTC GCC GCC GCG CTG CGC ACC GGC      394
Thr Ala Val Glu Ala Glu Asp Ala Val Ala Ala Ala Leu Arg Thr Gly
                100                 105                 110
CAG TTC AAC TGC TAC GCC GCC GGC GTC GGC CTC CCC GCC GCA CGA AGC      442
Gln Phe Asn Cys Tyr Ala Ala Gly Val Gly Leu Pro Ala Ala Arg Ser
            115                 120                 125
GCC GTA GCA GAG CAC TTG TCA CAG GGC GTG CCC TAC AAG CTA TCG GCC      490
Ala Val Ala Glu His Leu Ser Gln Gly Val Pro Tyr Lys Leu Ser Ala
        130                 135                 140
GAC GAC GTC TTC CTC ACC GCC GGC GGA ACT CAG GCG ATC GAA GTC ATA      538
Asp Asp Val Phe Leu Thr Ala Gly Gly Thr Gln Ala Ile Glu Val Ile
    145                 150                 155
ATC CCG GTG CTG GCC CAG ACT GCC GGC GCC AAC ATA CTG CTT CCC CGG      586
Ile Pro Val Leu Ala Gln Thr Ala Gly Ala Asn Ile Leu Leu Pro Arg
160                 165                 170                 175
CCA GGC TAT CCA AAT TAC GAG GCG CGA GCG GCA TTC AAC AAG CTG GAG      634
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Pro Gly Tyr Pro Asn Tyr Glu Ala Arg Ala Ala Phe Asn Lys Leu Glu
                180                 185                 190
GTC CGG CAC TTC GAC CTC ATC CCC GAC AAG GGG TGG GAG ATC GAC ATC      682
Val Arg His Phe Asp Leu Ile Pro Asp Lys Gly Trp Glu Ile Asp Ile
            195                 200                 205
GAC TCG CTG GAA TCC ATC GCC GAC AAG AAC ACC ACC GCG ATG GTC ATC      730
Asp Ser Leu Glu Ser Ile Ala Asp Lys Asn Thr Thr Ala Met Val Ile
        210                 215                 220
ATA AAC CCA AAC AAT CCG TGC GGC AGC GTT TAC TCC TAC GAC CAT CTG      778
Ile Asn Pro Asn Asn Pro Cys Gly Ser Val Tyr Ser Tyr Asp His Leu
    225                 230                 235
GCC AAG GTC GCG GAG GTG GCA AGG AAG CTC GGA ATA TTG GTG ATC GCT      826
Ala Lys Val Ala Glu Val Ala Arg Lys Leu Gly Ile Leu Val Ile Ala
240                 245                 250                 255
GAC GAG GTT TAC GGC AAA CTG GTT CTG GGC AGC GCC CCG TTT ATC CCG      874
Asp Glu Val Tyr Gly Lys Leu Val Leu Gly Ser Ala Pro Phe Ile Pro
                260                 265                 270
ATG GGC GTC TTT GGG CAC ATT GCC CCG GTC TTG TCC ATT GGA TCT CTG      922
Met Gly Val Phe Gly His Ile Ala Pro Val Leu Ser Ile Gly Ser Leu
            275                 280                 285
TCC AAG TCG TGG ATA GTG CCT GGA TGG CGA CTT GGA TGG GTG GCG GTG      970
Ser Lys Ser Trp Ile Val Pro Gly Trp Arg Leu Gly Trp Val Ala Val
        290                 295                 300
TAC GAC CCC ACA AAG ATT TTA GAG AAA ACT AAG ATC TCT ACG TCT ATT     1018
Tyr Asp Pro Thr Lys Ile Leu Glu Lys Thr Lys Ile Ser Thr Ser Ile
    305                 310                 315
ACG AAT TAC CTT AAT GTC TCA ACG GAC CCA GCA ACC TTC GTT CAG GAA     1066
Thr Asn Tyr Leu Asn Val Ser Thr Asp Pro Ala Thr Phe Val Gln Glu
320                 325                 330                 335
GCT CTT CCT AAA ATT CTT GAG AAC ACA AAA GCA GAT TTC TTT AAG AGG     1114
Ala Leu Pro Lys Ile Leu Glu Asn Thr Lys Ala Asp Phe Phe Lys Arg
                340                 345                 350
ATT ATT GGT CTA CTA AAG GAA TCA TCA GAG ATA TGT TAT AGG GAA ATA     1162
Ile Ile Gly Leu Leu Lys Glu Ser Ser Glu Ile Cys Tyr Arg Glu Ile
            355                 360                 365
AAG GAA AAC AAA TAT ATT ACG TGT CCT CAC AAG CCA GAA GGA TCG ATG     1210
Lys Glu Asn Lys Tyr Ile Thr Cys Pro His Lys Pro Glu Gly Ser Met
        370                 375                 380
TTT GTA ATG GTC AAA CTA AAC TTA CAT CTT TTG GAG GAG ATC CAT GAC     1258
Phe Val Met Val Lys Leu Asn Leu His Leu Leu Glu Glu Ile His Asp
    385                 390                 395
GAC ATA GAT TTT TGC TGC AAG CTC GCA AAG GAA GAA TCA GTA ATT TTA     1306
Asp Ile Asp Phe Cys Cys Lys Leu Ala Lys Glu Glu Ser Val Ile Leu
400                 405                 410                 415
TGT CCA GGG AGT GTT CTT GGA ATG GAA AAT TGG GTC CGT ATT ACT TTT     1354
Cys Pro Gly Ser Val Leu Gly Met Glu Asn Trp Val Arg Ile Thr Phe
                420                 425                 430
GCC TGC GTT CCA TCT TCT CTT CAA GAT GGA CTC GAA AGG GTC AAA TCA     1402
Ala Cys Val Pro Ser Ser Leu Gln Asp Gly Leu Glu Arg Val Lys Ser
            435                 440                 445
TTC TGT CAA AGG AAC AAG AAG AAG AAT TCT ATA AAT GGT TGT TAG         1447
Phe Cys Gln Arg Asn Lys Lys Lys Asn Ser Ile Asn Gly Cys
        450                 455                 460 461
TTGTACACAC CCCTAGTTGT ACATCTGACT GAAGCTGTAA ATCATTTCTA GTTATCCCCC   1507
ATTTATATAT TTCAATAAAA CATATTGTAA TGGTTCTGTT GTAGCTGTCC AAGTCATGTA   1567
CTCTACTTTT TGATGTATTT GGCCTCATTG CCTTGCATCA ATTTCAATAA AAATGGTTGT   1627
GTACACCAAA AAAAAAAAAA AAAAAAAAAA AAA                                1660
```

(5) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISICS:
(A) LENGTH: 1910 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY:
(ii) MOLECULAR TYPE: cDNA to mRNA
(iii) HYPOTHETICAL:
(iv) ANTI-SENSE:
(v) FEATURE: CDS
(vi) LOCATION: 76 .. 1731
(vii) IDENTIFICATION METHOD: P
(viii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
CGCGCTACTA GTAGTATTCC TGGTGTAGTC TAGTAGTACT CTCCTCCTCC TCCTTCTCCT      60
CCTACCCGTT TCCTC ATG GCC ACC GTA CGC CAG AGC GAC GGA GTC GCC GCG      111
                 Met Ala Thr Val Arg Gln Ser Asp Gly Val Ala Ala
                   1               5                  10
AAC GGC CTT GCC GTG GCC GCA GCC GCG AAC GGC AAG AGC AAC GGC CAT      159
Asn Gly Leu Ala Val Ala Ala Ala Ala Asn Gly Lys Ser Asn Gly His
          15                  20                  25
GGC GTG GCT GCC GCC GTG AAC GGC AAG AGC AAC GGC CAT GGC GTG GAT      207
Gly Val Ala Ala Ala Val Asn Gly Lys Ser Asn Gly His Gly Val Asp
      30                  35                  40
GCC GAC GCG AAC GGC AAG AGC AAC GGC CAT GGC GTG GCT GCC GAC GCG      255
Ala Asp Ala Asn Gly Lys Ser Asn Gly His Gly Val Ala Ala Asp Ala
45                  50                  55                  60
AAC GGC AAG AGC AAC GGC CAT GCC GAG GCC ACT GCG AAC GGC CAC GGC      303
Asn Gly Lys Ser Asn Gly His Ala Glu Ala Thr Ala Asn Gly His Gly
                  65                  70                  75
GAG GCC ACT GCG AAC GGC AAG ACC AAC GGC CAC CGC GAG AGC AAC GGC      351
Glu Ala Thr Ala Asn Gly Lys Thr Asn Gly His Arg Glu Ser Asn Gly
              80                  85                  90
CAT GCT GAG GCC GCC GAC GCG AAC GGC GAG AGC AAC GAG CAT GCC GAG      399
His Ala Glu Ala Ala Asp Ala Asn Gly Glu Ser Asn Glu His Ala Glu
          95                  100                 105
GAC TCC GCG GCG AAC GGC GAG AGC AAC GGG CAT GCG GCG GCG GCG GCA      447
Asp Ser Ala Ala Asn Gly Glu Ser Asn Gly His Ala Ala Ala Ala Ala
      110                 115                 120
GAG GAG GAG GAG GCG GTG GAG TGG AAT TTC GCG GGT GCC AAG GAC GGC      495
Glu Glu Glu Glu Ala Val Glu Trp Asn Phe Ala Gly Ala Lys Asp Gly
125                 130                 135                 140
GTG CTG GCG GCG ACG GGG GCG AAC ATG AGC ATC CGG GCG ATA CGG TAC      543
Val Leu Ala Ala Thr Gly Ala Asn Met Ser Ile Arg Ala Ile Arg Tyr
                  145                 150                 155
AAG ATC AGC GCG AGC GTG CAG GAG AAG GGG CCG CGG CCC GTG CTG CCG      591
Lys Ile Ser Ala Ser Val Gln Glu Lys Gly Pro Arg Pro Val Leu Pro
              160                 165                 170
CTG GCC CAC GGG GAC CCG TCC GTG TTC CCG GCC TTC CGC ACG GCC GTC      639
Leu Ala His Gly Asp Pro Ser Val Phe Pro Ala Phe Arg Thr Ala Val
          175                 180                 185
GAG GCC GAG GAC GCC GTC GCC GCC GCC GTG CGC ACC GGC CAG TTC AAC      687
Glu Ala Glu Asp Ala Val Ala Ala Ala Val Arg Thr Gly Gln Phe Asn
      190                 195                 200
TGC TAC CCC GCC GGC GTC GGC CTC CCC GCC GCA CGA AGC GCC GTG GCA      735
Cys Tyr Pro Ala Gly Val Gly Leu Pro Ala Ala Arg Ser Ala Val Ala
205                 210                 215                 220
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GAG CAC CTG TCG CAG GGC GTG CCG TAC ATG CTA TCG GCC GAC GAC GTC      783
Glu His Leu Ser Gln Gly Val Pro Tyr Met Leu Ser Ala Asp Asp Val
                225                 230                 235

TTC CTC ACC GCC GGC GGG ACC CAG GCG ATC GAG GTC ATA ATC CCG GTG      831
Phe Leu Thr Ala Gly Gly Thr Gln Ala Ile Glu Val Ile Ile Pro Val
            240                 245                 250

CTG GCC CAG ACC GCC GGC GCC AAC ATT CTG CTC CCC AGG CCA GGC TAC      879
Leu Ala Gln Thr Ala Gly Ala Asn Ile Leu Leu Pro Arg Pro Gly Tyr
        255                 260                 265

CCA AAC TAC GAG GCG CGC GCC GCG TTC AAC AGG CTG GAG GTC CGG CAT      927
Pro Asn Tyr Glu Ala Arg Ala Ala Phe Asn Arg Leu Glu Val Arg His
    270                 275                 280

TTC GAC CTC ATC CCC GAC AAG GGG TGG GAG ATC GAC ATC GAC TCG CTG      975
Phe Asp Leu Ile Pro Asp Lys Gly Trp Glu Ile Asp Ile Asp Ser Leu
285                 290                 295                 300

GAA TCC ATC GCC GAC AAG AAC ACC ACC GCC ATG GTC ATC ATA AAC CCC     1023
Glu Ser Ile Ala Asp Lys Asn Thr Thr Ala MeT Val Ile Ile Asn Pro
                305                 310                 315

AAC AAC CCG TGC GGC AGC GTT TAC TCC TAC GAC CAT CTG TCC AAG GTC     1071
Asn Asn Pro Cys Gly Ser Val Tyr Ser Tyr Asp His Leu Ser Lys Val
            320                 325                 330

GCG GAG GTG GCG AAA AGG CTC GGA ATA TTG GTG ATT GCT GAC GAG GTA     1119
Ala Glu Val Ala Lys Arg Leu Gly Ile Leu Val Ile Ala Asp Glu Val
        335                 340                 345

TAC GGC AAG CTG GTT CTG GGC AGC GCC CCG TTC ATC CCA ATG GGA GTG     1167
Tyr Gly Lys Leu Val Leu Gly Ser Ala Pro Phe Ile Pro Met Gly Val
    350                 355                 360

TTT GGG CAC ATC ACC CCT GTG CTG TCC ATA GGG TCT CTG TCC AAG TCA     1215
Phe Gly His Ile Thr Pro Val Leu Ser Ile Gly Ser Leu Ser Lys Ser
365                 370                 375                 380

TGG ATA GTG CCT GGA TGG CGG CTT GGA TGG GTA GCG GTG TAC GAC CCC     1263
Trp Ile Val Pro Gly Trp Arg Leu Gly Trp Val Ala Val Tyr Asp Pro
                385                 390                 395

AGA AAG ATC TTA CAG GAA ACT AAG ATC TCT ACA TCA ATT ACG AAT TAC     1311
Arg Lys Ile Leu Gln Glu Thr Lys Ile Ser Thr Ser Ile Thr Asn Tyr
            400                 405                 410

CTC AAT GTC TCG ACA GAC CCA GCA ACC TTC ATT CAG GCA GCT CTT CCT     1359
Leu Asn Val Ser Thr Asp Pro Ala Thr Phe Ile Gln Ala Ala Leu Pro
        415                 420                 425

CAG ATT CTT GAG AAC ACA AAG GAA GAT TTC TTT AAG GCG ATT ATT GGT     1407
Gln Ile Leu Glu Asn Thr Lys Glu Asp Phe Phe Lys Ala Ile Ile Gly
    430                 435                 440

CTG CTA AAG GAA TCA TCA GAG ATA TGC TAC AAA CAA ATA AAG GAA AAC     1455
Leu Leu Lys Glu Ser Ser Glu Ile Cys Tyr Lys Gln Ile Lys Glu Asn
445                 450                 455                 460

AAA TAC ATT ACA TGT CCT CAC AAG CCA GAA GGA TCA ATG TTT GTC ATG     1503
Lys Tyr Ile Thr Cys Pro His Lys Pro Glu Gly Ser Met Phe Val Met
                465                 470                 475

GTG AAA CTG AAC TTA CAT CTT TTG GAG GAA ATA GAC GAT GAC ATT GAT     1551
Val Lys Leu Asn Leu His Leu Leu Glu Glu Ile Asp Asp Asp Ile Asp
            480                 485                 490

TTT TGC TGC AAG CTC GCA AAA GAA GAA TCA GTA ATC TTA TGC CCA GGG     1599
Phe Cys Cys Lys Leu Ala Lys Glu Glu Ser Val Ile Leu Cys Pro Gly
        495                 500                 505

AGT GTT CTT GGA ATG GCA AAC TGG GTC CGC ATT ACT TTT GCT TGT GTT     1647
Ser Val Leu Gly Met Ala Asn Trp Val Arg Ile Thr Phe Ala Cys Val
    510                 515                 520

CCA TCT TCT CTT CAA GAT GGT CTC GGA AGG ATC AAA TCA TTC TGT CAA     1695
```

```
Pro Ser Ser Leu Gln Asp Gly Leu Gly Arg Ile Lys Ser Phe Cys Gln
525                 530                 535                 540
AGG AAC AAG AAG AGA AAT TCG AGC GAT GAT TGC TAG TTGTATATCT          1741
Arg Asn Lys Lys Arg Asn Ser Ser Asp Asp Cys
                545                 550 551
GACTGAAGCT GTAAATCATT CCCAGTATCC CCATCTATAT CTTTCAATAA AATGGAACTT   1801
TTAGTTCTCT ATGAATAGAA GTCAACATCT CCTTGAATAT GTTCTGGTTG TTGTGGCCTG   1861
GACGAAACAT AGTGAATGTT ATGTTAGTGA AGTTAAAAAA AAAAAAAAA               1910
```

(6) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURES:

(A) NAME/KEY: modified_base

(B) LOCATION:3,6,21

(D) OTHER INFORMATION:/note= "Note=A is Inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCAGTAGART GGAAYTTYGC AMG 23

(7) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURES:

(A) NAME/KEY: modified_base

(B) LOCATION:9,15,18

(D) OTHER INFORMATION:/note= "Note=A is Inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCDATRTGAC CRAAAACACC 20

Claims

1. A protein comprising an amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase-activity.

2. A gene encoding the protein as defined in claim 1.

3. The gene according to claim 2, which has a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1 or 2.

4. The gene according to claim 3, which has a nucleotide nucleotide sequence represented by SEQ ID NO: 3 or 4.

5. A plasmid comprising the gene as defined in claim 2.

6. An expression plasmid comprising:

(1) a promoter capable of functioning in a host cell,

(2) the gene as defined in claim 2 and

(3) a terminator capable of functioning in a host cell, operably in the above described order.

7. A process for constructing an expression plasmid, which comprises combining:

(1) a promoter capable of functioning in a host cell,
(2) the gene as defined in claim 2 and
(3) a terminator capable of functioning in a host cell, operably in the above described order.

8. A transformant comprising a host cell harboring the plasmid as defined in claim 5 or 6.

9. The transformant according to claim 8, wherein the host is a microorganism.

10. The transformant according to claim 8, wherein the host cell is a plant cell,

11. A process for enhancing iron absorbing ability of a host cell, which comprises introducing into a host cell an expression plasmid formed by combining (1) a promoter capable of functioning in a host cell, (2) a nicotianamine aminotransferase gene and (3) a terminator capable of functioning in a host cell, operably in the above described order and transforming said host cell.

12. The process according to claim 11, wherein the host cell is a plant cell,

13. The process according to claim 12, wherein the gene of the nicotianamine aminotransferase is the gene as defined in claim 2.

14. A gene fragment having a partial sequence of the gene as defined in claim 2, 3 or 4.

15. The gene fragment according to claim 14, wherein the number of the base is 15 or more and 50 or less.

16. The gene fragment according to claim 14, which has the nucleotide sequence represented by SEQ ID NO: 5.

17. A process for detecting a nicotianamine aminotransferase gene, which comprises detecting from plant gene fragments a nicotianamine aminotransferase gene having a nucleotide sequence encoding an amino acid sequence of an enzyme with the nicotianamine aminotransferase activity or a gene fragment thereof by applying the hybridization method using the gene fragment as defined in claim 14, 15 or 16.

18. A process for amplifying a nicotianamine aminotransferase gene, which comprises amplifying a nicotianamine aminotransferase gene having a nucleotide sequence encoding an amino acid sequence of an enzyme with the nicotianamine aminotransferase activity or a gene fragment thereof by applying PCR (polymerase chain reaction) on a plant gene fragment using the gene fragment as defined in claim 14, 15 or 16 as a primer.

19. A process for obtaining a nicotianamine aminotransferase gene, which comprises identifying a nicotianamine aminotransferase gene or a gene fragment thereof by the process as defined in claim 17 or 18, and isolating and purifying the identified gene or the gene fragment thereof.

20. A nicotianamine aminotransferase gene obtained by the process as defined in claim 19.

(19) Europäisches Patentamt
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(54) Nicotianamine aminotrassferase and gene therefor

(57) A protein having an amino acid sequence represented by SEQ ID NO: 1 or 2 or an amino acid sequence having said amino acid sequence with a single or plural amino acids deleted, replaced or added, and having the nicotianamine aminotransferase activity, a gene encoding said protein as well as utilization thereof for enhancement of ability of absorbing insoluble iron in soil and for improvement of resistance to iron deficiency are provided.

EP 0 860 499 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 10 2891

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	S. MORI: "Reevaluation of the genes induced by iron deficiency in barley roots" SOIL SCI. PLANT NUTR., vol. 43, 1997, pages 975-980, XP002076369 *see the whole article*	1-20	C12N9/12 C12N15/63 C12N1/21 C12N5/10 C12N15/29
X	Plant nutrition for sustainable food production and environment. T. Ando eds. Kluwer Academic press. Doordrecht. 1997, p. 279-280. M. Takahashi et al. Purification, characterization and DNA sequencing of nicotianamine aminotransferase (NAAT-III) expressed in Fe- deficient barley roots. XP002076371 *see the whole article*	1-20	
A	K. KANAZAWA ET AL.: "Detection of two distincts isoenzymes of nicotianamine aminotransferase in Fe-deficient barley roots*see the whole article*" JOURNAL FOR EXPERIMENTAL BOTANY, vol. 46, no. 290, 1995, pages 1241-1244, XP002076370	1-20	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	3 September 1998	Marie, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
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E : earlier patent document, but published on, or after the filing date
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